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14. ABSTRACT This one-year project was aimed to establish a new animal model and generate preliminary data with the ultimate goal of ascertaining whether the post-operative tissue microenvironment, caused by breast conserving surgery, promotes metastatic dissemination. We engineered 4T1 murine mammary breast cancer cells to express either enhanced Green Fluorescent protein (eGFP) or Dendra2 green-to-red photoswitchable fluorescent protein. Furthermore, we established a mouse model of partial and complete resection of mammary tumors. Among the genes that we believe will be altered in breast cancer cells by the post-operative stroma is CX ₃ CR1, the sole receptor for the chemokine fractalkine (CX3CL1). We therefore investigated the role of this receptor in the metastatic dissemination of breast cancer to the skeleton and described for the first time its crucial role in cancer cell adhesion to bone marrow endothelium and extravasation into the bone stroma.					
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INTRODUCTION

Breast conserving surgery is an elective therapy for breast cancer. Unfortunately, many patients will eventually develop metastases, often years after initial treatment of the primary tumor. Metastasis is by far the main cause of death from breast cancer.

The detection of residual tumor after surgical intervention is a common occurrence following lumpectomy and the post-operative mammary stroma is characterized by the production of tissue factors that could alter the phenotype of residual cancer cells.

We hypothesize that post-operative changes in the stroma can accelerate the growth of cancer cells and possibly also increases the potential for metastatic dissemination.

This one-year project was aimed to establish an animal model that could be employed to identify specific genes that confer metastatic potential to breast cancer cells and are altered by the post-operative stroma following breast-conserving surgery.

BODY

During the first period of the funded work, we engineered 4T1 murine breast cancer cells to stably express the photoswitchable fluorescent protein Dendra2. The illumination of these cells with UV-light (Dymax BlueWave) induces a switch in emitted fluorescence from the green spectrum (510-530nm) to the red spectrum (570-610nm), as shown in **figure 1**.

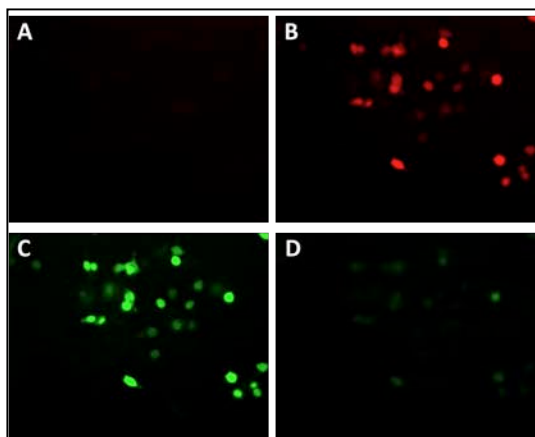


Figure 1. The photo switch of Dendra2 in 4T1 breast cancer cells. Before illumination with UV-light, the cells showed negligible red fluorescence coupled to intense fluorescence in the green spectrum (A and C). Following illumination, red fluorescence increased dramatically while the green emission shows a significant reduction (B and D)

When inoculated in the mammary fat pad of female Balb/c mice (7×10^3 in 100 μ l), these cells produce tumors that can then be partially or totally excised (**fig.2**).

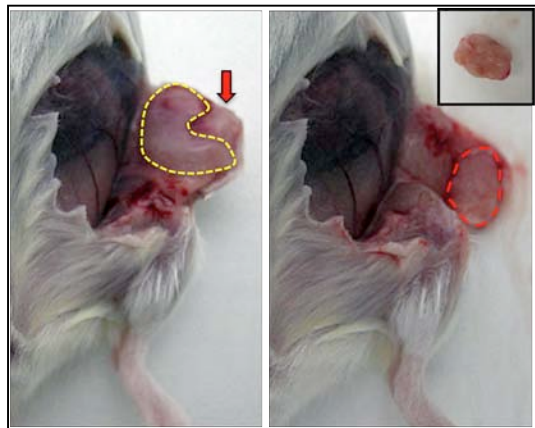


Figure 2. Animal model of breast cancer partial resection. Mammary fat pad tumors were produced by murine 4T1 breast cancer cells inoculated in female Balb/c mice. Upon producing a semielliptical incision, the skin flap reveals a tumor (dotted yellow line) that can be partially excised (inset). The residual tumor is delineated by the red dotted line.

The microscopic analysis of the tumor tissue surgically removed as described above allows the identification of cancer cells infiltrating the stroma by the detection of their green fluorescence (**fig. 3**).

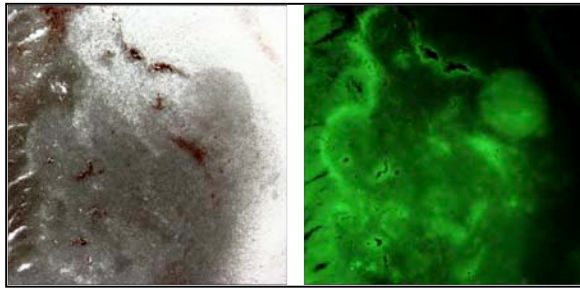


Figure 3. Green fluorescent tumors generated by 4T1 cells expressing Dendra2 protein. Tumor tissue was fixed and rapidly frozen. Cryosections of 20µm in thickness were obtained and inspected with a fluorescence stereomicroscope.

We are currently conducting experiments in which the residual tumors are UV-light illuminated to induce the photoswitch of the Dendra2 protein.

Simultaneously with the experiments described above, we also developed a parallel line of investigation of paramount importance for the positive outcome of the study.

This proposal conceptually revolves around the hypothesis that the widespread idea that the emergence of metastatic disease is entirely determined by events already occurred when surgery is performed – and that are therefore no longer preventable – might not fully represent the actual clinical scenario.

We therefore propose that metastatic dissemination might take place also *after* primary therapeutic intervention and be caused by cancer cells departing from either residual tumor or recurrences. Local surgery on the primary mammary tumor induces the production of a plethora of wound factors that profoundly affect the proliferation of cancer cells ¹ and could affect cells that were not eliminated by surgical excision. Prior to re-intervention, residual cancer cells in patients with positive resection margins may benefit from a fertile stromal environment that promotes dissemination.

The ultimate goal of this study is therefore the identification of specific genes or gene signatures that confer metastatic potential to breast cancer cells and are regulated by the changes in the stroma microenvironments surrounding the residual tumor cells following conserving breast cancer surgery

The gene coding for CX3CR1, the sole receptor for the chemokine fractalkine (FKN, CX3CL1) is of particular interest for the dissemination of cancer cells to the skeleton. The arrest of circulating cancer cells to the skeleton is highly dependent on specific adhesive interactions with the endothelial cells lining the marrow sinusoids ^{2 3 4}. The required next step is the extravasation of adherent cancer cells drawn by chemoattractant cues generated by the surrounding stroma ⁵. FKN exists as a trans-membrane protein that is cleaved into a soluble molecule with potent chemoattractant properties ⁶. In its membrane-bound form, FKN can establish strong and stable adhesive interactions with its receptor CX₃CR1, and does not require any downstream signaling to induce activation of additional adhesion molecules ^{7 8 9}. Because of its

unique properties, FKN is an ideal candidate to mediate both adhesion and extravasation of CX₃CR1-bearing circulating cancer cells.

We were the first to report that cancer cells expressing CX₃CR1 on their surface adhere to human bone marrow endothelial cells under dynamic-flow conditions in a FKN-dependent manner¹⁰.

A necessary step to be undertaken was to ascertain whether human breast expresses the chemokine receptor. To this end, we processed human tissue microarrays of normal and malignant mammary glands for CX₃CR1 detection by immunohistochemistry. As shown by **figure 4**, both normal and tumor tissues stained diffusely for CX₃CR1 and the signal was strictly limited to the epithelial compartment. However, 26% of malignant mammary glands showed the strongest staining intensity and broader signal distribution, which was observed only in 1 out of 47 samples on normal mammary tissue (**Table 1**).

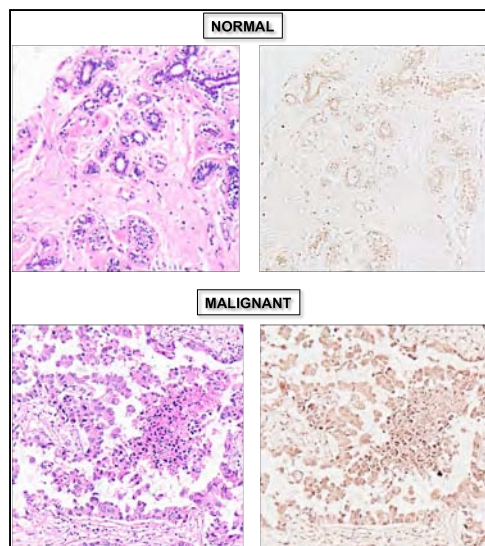


Figure 4. Immunohistochemistry detection of CX₃CR1 in human breast tissue arrays. Hematoxylin and eosin staining (left) and immunohistochemistry detection of CX₃CR1 (right) in human mammary glands. A positive staining for the chemokine receptor could be observed in epithelial cells from both normal (and malignant breast tissue. The stromal component stained uniformly negative for CX₃CR1. Original magnification, x50.

Tissues	0	0-1	1-2	2-3	Total
Normal	21	19	6	1	47
	(45%)	(40%)	(13%)	(2%)	
Malignant	43	54	53	52	202
	(21%)	(27%)	(26%)	(26%)	

Intensity and distribution of CX₃CR1 in normal and malignant breast tissues.

Staining intensity was scored based on a scale in which negative specimens were graded (0) and strongly positive samples were graded (3). To evaluate distribution, tissues with complete lack of staining were scored as (0); tissues prevalently negative for CX₃CR1 displaying some areas of positive staining were scored as (0-1); tissues showing uniform staining for CX₃CR1 were scored as either (1-2) or (2-3), based on the intensity of the signal observed.

Of the 202 malignant samples examined, only 4 were identified as being Ductal Carcinoma In Situ (DCIS): two were scored as (0) and the other two as (0-1) and (1-2). The increase in CX₃CR1 expression observed in cancer samples as compared to normal samples resulted to be very statistically significant with a p value equal to 0.0016.

Because of the strong propensity to disseminate and grow in the bone shown by breast adenocarcinoma, we asked whether a correlation could be found between the levels of CX₃CR1 expression and bone-metastatic potential of human breast cancer cell lines. Thus, we selected MDA-231 cells, which are widely recognized as strongly bone-metastatic^{11 12 13}, and MDA-436 cells for which the ability to efficiently target the skeleton and grow into macroscopic metastases in animal models has never been reported. When these two cell lines were tested for CX₃CR1 by western blotting, MDA-

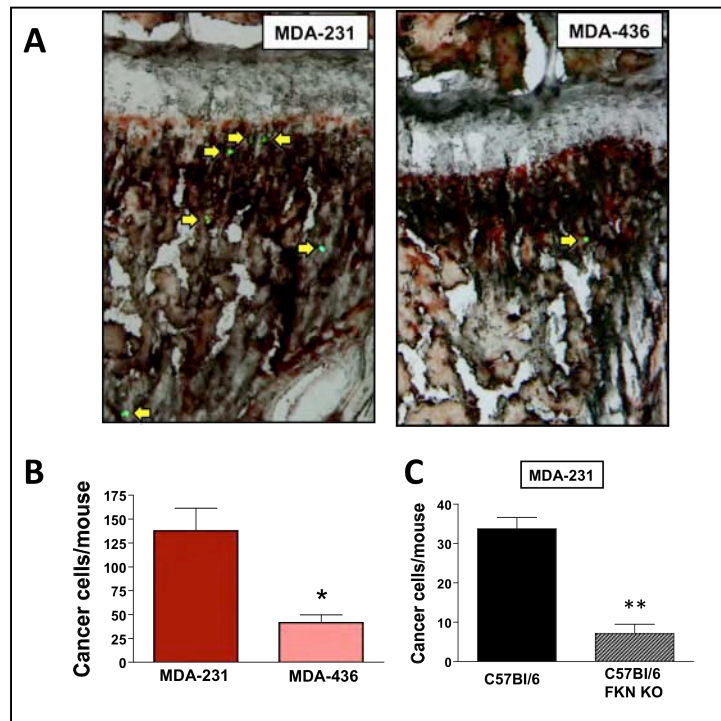


Figure 4. The arrest of breast cancer cells to the skeleton depends on CX₃CR1 expression. Single breast cancer cells stably expressing eGFP (arrows) were identified by fluorescence stereomicroscopy in the bone marrow of mice, 24 hours after being inoculated via the left cardiac ventricle (**A**); MDA-231 and MDA-436 cell lines that migrated to the skeleton via the hematogenous route, expressed as mean of cells detected per mouse \pm S.E.M. (**B**). (MDA-231 cells = 4 mice, MDA-436 cells = 8 mice. * $p=0.0008$); The number of MDA-231 cells detected in the skeleton of transgenic FKN(-/-) mice (n=5) 24 hours after intra-cardiac inoculation is significantly decreased compared to the number of cells detected in normal C56Bl/6 mice (n=4) (**C**). **- $p<0.0001$.

231 cells were found positive whereas MDA-436 express minimal levels of this receptor (please refer to the original manuscript in the appendix). To investigate whether this disparity in CX₃CR1 expression could account for a different ability to arrest at the skeleton, we tested these two breast cancer cell lines by delivering them into the blood circulation of mice. As previously described, we have recently established an animal model of metastatic dissemination that allows the identification of single cancer cells immediately after their arrival to different tissue, including the bone¹⁴. Thus, Balb/c mice were inoculated via the left cardiac ventricle with MDA-231 or MD-436 cells that stably expressed eGFP and sacrificed 24 hours later. The tibiae and femora of each animal were collected, processed for cryosectioning and inspected by fluorescence microscopy. Disseminated cancer cells were identified and counted throughout each entire bone by analyzing all serial sections obtained. These experiments revealed a much stronger tendency of MDA-231 cells to homing to the bone marrow than MDA-436 cells (**Fig.5 A,B**). The 3-fold higher number of MDA-231 cells detected in the bone of inoculated mice correlates with the higher expression of CX₃CR1

detected in these cells as compared to MDA-436 cells.

A more causal evidence for the role exerted by the CX₃CR1-FKN pair in breast cancer cell lodging to the bone would require interfering with the molecular interactions between the chemokine and its receptor *in vivo*. This opportunity was offered by a FKN-null transgenic mouse previously generated by Dr. Lira and collaborators¹⁵. FKN(-/-) mice inoculated with CX₃CR1-expressing MDA-231 cells displayed a dramatic reduction of more than 70% in the number of breast cancer cells detected 24 hours later in the bone marrow of tibia and femur, as compared to wild-type animals (**Fig. 5 C**).

Finally, we were able to dissect and characterize the different contribution provided by CX₃CR1 in either adhesion or extravasation of breast cancer cells targeting the skeleton (**please refer to the original manuscript in the appendix**).

KEY RESEARCH ACCOMPLISHMENTS

The one-year funding provided by this Concept award has allowed us to:

- a) Generate cell lines and establish animal models that will be used for studying the post-operative stroma and its role in promoting metastatic dissemination of breast cancer cells. These newly acquired tools will be invaluable for continuing our investigation focused on the effects of the surgical wound on residual cancer cells and metastatic disease.
- b) Identify – for the first time – a direct correlation between the expression of CX₃CR1 and the bone metastatic potential of breast cancer cells. More importantly, the use of transgenic mice null for the chemokine FKN allowed us to provide a conclusive demonstration that CX₃CR1-FKN interactions are responsible for adhesive and extravasation events involved in skeletal metastasis from breast cancer.
- c) Recognize CX₃CR1 as a crucial gene to be monitored in gene-expression comparative studies including cancer cells isolated from primary breast tumors and their local recurrences following surgical intervention.
- d) Identify CX₃CR1 as a potential pharmacological target for therapeutic strategies directed to protect breast cancer patients from metastatic dissemination in the post-operative period, before the adoption of post-adjuvant therapies.

REPORTABLE OUTCOMES

Papers

- The chemokine receptor CX₃CR1 is directly involved in the arrest of breast cancer cells to the skeleton. Whitney L. Jamieson, Yun Zhang, Alan M. Fong, Olimpia Meucci and Alessandro Fatatis. **Cancer Research** (*accepted for publication*) 2010.

Abstracts

- Fractalkine and CX₃CR1 are involved in the early stages of skeletal metastasis. Whitney Jamieson, Karim Dorgham, Philippe Deterre and Alessandro Fatatis. 101st Annual Meeting of the American Association for Cancer Research. Washington, DC., 2010.

- The chemokine fractalkine and its receptor CX₃CR1 are directly involved in the arrest of circulating breast and prostate cancer cells to the skeleton. Yun Zhang, Whitney L. Jamieson and Alessandro Fatatis. Joint Metastasis Research Society-AACR conference. Philadelphia, PA, 2010.

Presentations

- 2009 Kimmel Cancer Center – Thomas Jefferson University.

“Molecules and events implicated in skeletal metastasis from prostate cancer”. Philadelphia. PA.

- 2010 University of Florida. Shands Cancer Center

“Molecules and signaling events implicated in skeletal metastasis from prostate cancer”. Gainesville, FL.

Ph.D awarded

- Whitney Jamieson, degree awarded in April 2010

CONCLUSIONS

Based on our study, it seems reasonable to postulate that the dissemination to the skeleton of breast cancer cells can be effectively counteracted by interfering with the molecular and functional interactions between the chemokine FKN and its only receptor CX₃CR1. The expression of this receptor could be regulated by tissue factors locally produced in the mammary stroma during the post-operative period.

The studies described in this report, made possible by the one-year Concept Award funding, represent a prelude to a much broader investigation that will be conducted based on the preliminary evidence produced and cellular and animal models that were generated during the funded period.

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APPENDICES

THE CHEMOKINE RECEPTOR CX₃CR1 IS DIRECTLY INVOLVED IN THE ARREST OF BREAST CANCER CELLS TO THE SKELETON.

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Running title: CX₃CR1 and skeletal metastasis from breast cancer.

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ABSTRACT

Skeletal metastases from breast adenocarcinoma are responsible for most of the morbidity and mortality associated with this tumor and represent a significant and unsolved problem for therapy. The arrival of circulating cancer cells to the skeleton depends first on the adhesive interactions with the endothelial cells lining the bone marrow sinusoids, and then the extravasation toward chemoattractant molecules produced by the surrounding bone stroma.

We have previously shown that the membrane-bound and cell-adhesive form of the chemokine fractalkine is exposed on the luminal side of human bone marrow endothelial cells and that bone stromal cells release the soluble and chemoattractant form of this chemokine.

This study provides compelling evidence for the adhesive and chemotactic interactions between the CX₃CR1 receptor expressed by breast cancer cells and fractalkine in the bone tissue. We have detected CX₃CR1 protein in tissue microarrays of normal and malignant mammary glands. Using a pre-clinical animal model of hematogenous metastasis we show that breast cancer cells expressing high levels of this receptor have higher propensity to disseminate to the skeleton. Furthermore, studies with fractalkine-null transgenic mice demonstrated that the ablation of the adhesive and chemotactic ligand of CX₃CR1 dramatically impairs the skeletal dissemination of breast cancer cells. Finally, we used cells that were engineered to express exogenous wild type or functional mutants of CX₃CR1 to conclusively confirm the determinant role of this receptor in the arrest of breast cancer cells to the skeleton.

INTRODUCTION

Currently, only six percent of women that are first diagnosed with breast adenocarcinoma present with metastases.¹ Unfortunately, between twenty and fifty percent of them will eventually develop metastatic disease¹. Metastases are responsible for an intolerably high number of deaths among patients that would otherwise be almost invariably cured by surgical resection and adjuvant therapy². Autopsy studies have estimated that 70% of advanced breast cancer patients have skeletal metastases³. These secondary bone tumors cause significant morbidity, leading to considerable pain, spinal cord compression and pathological fractures⁴. In addition, when breast cancer cells have disseminated to the skeleton, the resulting bone tumors can be treated only with palliative measures^{5 6 7}.

The majority of patients develop metastases years after initial treatment of the primary breast tumor. The appearance of late metastases can indeed be attributed to cancer cells disseminated to secondary tissues during different stages of primary tumor progression and remained dormant for variable periods of time. In fact, both early dissemination and dormancy of tumor cells are supported by strong evidence^{8 9}. However, the concept that the emergence of metastatic disease is entirely determined by events already occurred when surgery is performed – and that are therefore no longer preventable – might not fully represent the actual clinical scenario. Metastatic dissemination might take place also *after* primary therapeutic intervention and be caused by cancer cells departing from either residual tumor or recurrences. For instance, the detection of positive surgical margins upon resection of breast tumors is a common occurrence and is directly related to the incidence of tumor recurrence^{10 11 1}. Local surgery on the primary mammary tumor induces the production of a plethora of wound factors that profoundly affect the proliferation of cancer cells¹² and could affect cells that were not eliminated by surgical excision. Prior to re-intervention, residual cancer cells in patients with positive resection margins may benefit from a fertile stromal environment that promotes dissemination. This

process would produce secondary waves of micrometastases with - at least - equal probability of developing into macroscopic tumors as those seeded years earlier. Thus, the adoption of adjuvant measures aimed to interfere with the arrival of cancer cells to the skeleton would protect breast cancer patients from post-surgery tumor dissemination.

The arrest of circulating cancer cells to the skeleton is highly dependent on specific adhesive interactions with the endothelial cells lining the marrow sinusoids ^{13 14 15}. The required next step is the extravasation of adherent cancer cells drawn by chemo attractant cues generated by the surrounding stroma ¹⁶. The similarities between cancer cell dissemination and leukocyte trafficking lead to the identification of chemokines as crucial players in both sets of events ¹⁷. The interactions between the chemokine CXCL12 (SDF-1) and its receptor CXCR4 have been extensively studied ^{18 19}. The role of CXCR4 in cell adhesion appears to be dependent on the secondary induction of $\alpha_v\beta_3$ integrin presentation on the surface of cancer cells and consequent binding to vascular adhesion molecules ²⁰. However, CXCR4 inhibition did not block the binding of colon cancer cells to the liver endothelium, but did limit extravasation ²¹. Thus, similarly to its role in hematopoietic stem cells homing, the soluble chemokine CXCL12 seems to be an important player in cancer cell migration into the bone microenvironment rather than mediating adhesive interactions with CXCR4-bearing cells ²². The chemokine CX3CL1 (Fractalkine or FKN, which will be used throughout the rest of the manuscript), on the other hand, exists as a trans-membrane protein that is cleaved into a soluble molecule with potent chemoattractant properties ²³. In its membrane-bound form, FKN can establish strong and stable adhesive interactions with its receptor CX₃CR1, and does not require any downstream signaling to induce activation of additional adhesion molecules ^{24 25 26}. Because of its unique properties, FKN is an ideal candidate to mediate both adhesion and extravasation of CX₃CR1-bearing circulating cancer cells.

We were the first to report that prostate cancer cells express CX₃CR1 and that these cells, under dynamic-flow conditions, adhere to human bone marrow

endothelial cells in a FKN-dependent manner²⁷. In addition, we have shown that CX₃CR1 is expressed in a high percentage of prostate cancer tissues while human bone marrow supernatants contain soluble FKN, which is released from cells of the bone stroma through a mechanism regulated by androgens²⁸.

A role for the FKN/CX₃CR1 pair in metastasis is also supported by the work of Andre and colleague showing a correlation between CX₃CR1 expression in primary breast tumors and clinical metastases. In addition, CX₃CR1 expression in pancreatic tumor cells was found to promote the infiltration of the central nervous system²⁹. Finally, Nevo and colleagues have recently reported that FKN and CX₃CR1 are involved in adhesion of neuroblastoma cells to the bone in an *in vitro* system³⁰.

Here we show that both normal and malignant breast tissues express CX₃CR1 and the ability of breast cancer cells to lodge in the skeleton of animal models is increased by the over-expression of CX₃CR1. Remarkably, when breast cancer cells were inoculated in transgenic FKN knockout mice via a hematogeneous route, a seventy percent reduction in the detection of bone-disseminated cells was observed as compared to FKN-expressing animals. Finally, by using functional mutants of CX₃CR1 we provide evidence that this receptor regulates both adhesion and extravasation of breast cancer cells.

MATERIALS AND METHODS

Cell lines and cell cultures. MDA-MB-231 (MDA-231) and MDA-MB-436 (MDA-436) human breast cancer cells were purchased from ATCC (Manassas, VA). The PC3-ML sub-line was derived from the parental PC-3 cells as previously described³¹. All cells were grown in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT) and 0.1% gentamicin (Invitrogen, Carlsbad, CA) and kept at 37 °C and 5% CO₂. For the experiments performed *in vivo*, cells were engineered to stably express enhanced Green Fluorescent Protein (eGFP) using a lentiviral vector from America Pharma Source (Bethesda, MD).

Transfection and selection of stable cell lines. The cDNAs for wild type and mutant CX₃CR1 isoforms were inserted in the pEGFP-N1 vector (Clontech, Inc.,

Mountain View, CA). MDA-436 were transfected with 3 µg of plasmid DNA using the Lipofectamine 2000 transfection system according to manufacturer's instructions (Invitrogen). Stable transfected cells were selected using geneticin (Invitrogen).

Immunohistochemistry and tissue array analysis. Three different breast tissue microarrays (BRC1502, BR1002 and BR722) were obtained from US Biomax (Rockville, MD) and the staining for CX₃CR1 was performed as described previously ²⁸, with an antibody against CX₃CR1 (7201) obtained from Abcam (Cambridge, MA) and used at a 3.3µg/ml concentration. In total, 202 tissue cores of breast cancer and 47 cores of normal breast tissue were examined.

Animal models of metastasis. Five week-old female CB17-SCID, C57Bl/6 and Balb/c mice were obtained from Taconic (Germantown, NY) and housed in a germ-free barrier. C57Bl/6-FKN^{-/-} transgenic mice were obtained from Schering-Plough (now Merck-Schering Plough, Whitehouse Station, NJ) and bred in house. Balb/c and CB17-SCID mice were used for the detection of bone-disseminated breast cancer cells at 24 and 72 hours post-inoculation, respectively. C57Bl/6 mice were used as same-strain controls for the C57Bl/6-FKN^{-/-} transgenic mice to detect cancer cells disseminated to the skeleton at 24 hours post-inoculation. At 6-8 weeks of age, mice were anesthetized with ketamine (80mg/kg) and xylazine (10mg/kg) and inoculated in the left cardiac ventricle with human cancer cells (5x10⁵ for MDA-436 and PC3-ML cells and 1x10⁵ for MDA-231 cells in a total volume of 100µl of DMEM/F12). Blue-fluorescent polystyrene beads (10µm diameter, Invitrogen-Molecular Probes) were co-injected to confirm successful delivery in the blood circulation. All experiments were performed in accordance with NIH guidelines for the humane use of animals. All protocols involving the use of animals were approved by the Drexel University College of Medicine Committee for the Use and Care of Animals.

Tissue preparation and cancer cell detection. Animals were sacrificed and tissues were fixed, decalcified in 0.5M EDTA if necessary and frozen in O.C.T.

embedding medium (Electron Microscopy Sciences, Hatfield, PA) as previously described ³². Serial tissue sections of 80µm in thickness were obtained using a Microm HM550 cryostat (Mikron, San Marcos, CA). Sections of each hind leg and soft-tissue organs were transferred on glass slides, stored at -20 °C and examined for cancer cells using either an Olympus IX70 fluorescence inverted microscope or an Olympus SZX12 fluorescence stereomicroscope. Bright field and fluorescence images were acquired with an Olympus DT70 CCD color camera.

Detection of soluble FKN in murine bone marrow. Bone marrow was flushed from the hind legs of wild type C57Bl/6 mice or FKN-null mice. The cellular component was removed by centrifugation at 2000 r.p.m. for 10 minutes at 4°C. Soluble FKN was detected using an ELISA DuoSet kit for murine FKN (R&D Systems) as previously described ²⁸.

CX₃CR1 signaling *in vitro*. Cells were serum starved for 4 hours and then exposed to 50nM recombinant human FKN (R&D systems, Minneapolis, MN) for indicated time points.

Cell surface CX₃CR1 protein isolation.

The amount of either wild-type or functional mutant forms of CX₃CR1 that were expressed by MDA-436 breast cancer cells at the plasma membrane level were measured by cell surface biotinylation, using a dedicated kit (cat. #89881) obtained from Pierce (Rockford, IL), according to the protocol provided by the manufacturer.

SDS-PAGE and western blotting. Cell lysates were obtained and SDS-polyacrylamide gel electrophoresis and western blot analysis were performed as previously described ²⁷, with few modifications. Membranes were probed with an antibody against CX₃CR1 (0.5µg/ml, Torrey Pines Biolabs, East Orange, NJ) and actin (Sigma, St. Louis, MO) using 5% milk as a blocking reagent. Membranes were also probed with antibodies targeting phospho-p44/42 MAPK (Thr202/Tyr204, Cell Signaling) and total p44/42 MAPK (Cell Signaling). All primary antibody incubations were performed overnight at 4°C. Primary antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit

secondary antibody (Pierce, Rockford, IL). Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce) and detected with the Fluorochem 8900 imaging system and relative software (Alpha Innotech, San Leandro, CA).

Statistics.

Statistical significance for the *in vivo* studies was determined using a one-tailed Student's T-test using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA) and data are presented as mean \pm standard error of the mean (S.E.M.). The significance of CX₃CR1 staining of TMAs was established using two-tailed Fisher's exact test performed using GraphPad Prism and using the method of summing small P values.

RESULTS AND DISCUSSION

This study was based on the working hypothesis that specific interactions between the receptor CX₃CR1 and its chemokine ligand FKN are responsible for the arrival and lodging of circulating breast cancer cells to the skeleton.

To ascertain whether human breast expresses the chemokine receptor, we processed human tissue microarrays of normal and malignant mammary glands for CX₃CR1 detection by immunohistochemistry. As shown by **fig. 1**, both normal and tumor tissues stained diffusely for CX₃CR1 and the signal was strictly limited to the epithelial compartment. However, 26% of malignant mammary glands showed the strongest staining intensity and broader signal distribution, which was observed only in 1 out of 47 samples on normal mammary tissue (**Table 1**). Thus, the CX₃CR1 chemokine receptor is frequently detected in normal mammary glands and its expression and distribution increases with malignant transformation. This scenario closely resembles what we previously described for human prostate tissue ²⁸. Our previous studies with human prostate cancer cell lines also revealed that the CX₃CR1-FKN pair mediates both the adhesion of cancer cells to endothelial cells of the bone marrow under dynamic-flow conditions and their migration following chemoattractant gradients *in vitro* ²⁷. These events are crucial to ensure the arrest and lodging of cancer cells

departing from the primary tumor and arriving to the skeleton through the hematogenous route. Because of the strong propensity to disseminate and grow in the bone shown by breast adenocarcinoma, we asked whether a correlation could be found between the levels of CX₃CR1 expression and bone-metastatic potential of human breast cancer cell lines. Thus, we selected MDA-231 cells, which are widely recognized as strongly bone-metastatic^{33 34 35}, and MDA-436 cells for which the ability to efficiently target the skeleton and grow into macroscopic metastases in animal models has never been reported. When these two cell lines were tested for CX₃CR1 by western blotting, MDA-231 cells were found positive whereas MDA-436 express minimal levels of this receptor (**Fig. 2A**). To investigate whether this disparity in CX₃CR1 expression could account for a different ability to arrest at the skeleton, we tested these two breast cancer cell lines by delivering them into the blood circulation of mice. As previously described, we have recently established an animal model of metastatic dissemination that allows the identification of single cancer cells immediately after their arrival to different tissue, including the bone³². Thus, Balb/c mice were inoculated via the left cardiac ventricle with MDA-231 or MD-436 cells that stably expressed eGFP and sacrificed 24 hours later. The tibiae and femora of each animal were collected, processed for cryosectioning and inspected by fluorescence microscopy. Disseminated cancer cells were identified and counted throughout each entire bone by analyzing all serial sections obtained. These experiments revealed a much stronger tendency of MDA-231 cells to homing to the bone marrow than MDA-436 cells (**Fig.2 B,C**). The 3-fold higher number of MDA-231 cells detected in the bone of inoculated mice correlates with the higher expression of CX₃CR1 detected in these cells as compared to MDA-436 cells. However, a more causal evidence for the role exerted by the CX₃CR1-FKN pair in breast cancer cell lodging to the bone would require interfering with the molecular interactions between the chemokine and its receptor *in vivo*. This opportunity was offered by a FKN-null transgenic mouse previously generated by Dr. Lira and collaborators³⁶. So far, this approach could not be pursued to test the role in metastasis of other chemokines, such as CXCL12/SDF1, since

CXCL12-null transgenic mice are not viable. In contrast, besides surviving targeted gene-disruption, FKN-null mice do not exhibit overt behavioral abnormalities or macroscopic anatomical alterations. The CX₃CR1-FKN pair is involved in leukocyte trafficking and immune response in general; an in depth analysis of these animals revealed that the responses to inflammatory stimuli were indistinguishable from those of C57Bl/6 wild-type mice. We used an ELISA-based assay and confirmed the absence of the soluble chemokine in the bone marrow of FKN(-/-) mice, which in contrast was detected in the parental C57Bl/6 mouse strain at an average concentration of 6.4ng/ml. FKN(-/-) mice inoculated with CX₃CR1-expressing MDA-231 cells displayed a dramatic reduction of more than 70% in the number of breast cancer cells detected 24 hours later in the bone marrow of tibia and femur, as compared to wild-type animals (**Fig. 3A**). To broaden the implications of this observation, we decided to replicate these experiments by inoculating FKN(-/-) mice with PC3-ML human prostate cancer cells, which also express high levels of CX₃CR1²⁷. This sub-line was previously isolated from the parental PC3 line³¹ and we found it to be highly bone-metastatic in animal models^{37 32 38 39}. Similarly to MDA-231 breast cancer cells, the arrival of PC3-ML cells to the bone through the blood circulation was reduced of more than 60% by the ablation of FKN expression (**Fig.3B**). These results, obtained with human cancer cells from two different types of solid tumors, provide the most compelling evidence to date that the interactions between CX₃CR1 and FKN regulate the initial homing of circulating cancer cells to the skeleton. As a small number of cells migrated to the bone in FKN(-/-) mice were still detected, it is plausible that additional molecules participate to the phenomenon. However, the dramatic reduction of cells lodged to the skeleton upon ablation of FKN indicates that CX₃CR1 expression should be considered an important feature of breast cancer cells with bone-metastatic potential. Interestingly, we observed no differences between wild-type and FKN(-/-) mice in the number of MDA-231 and PC3-ML cells detected in the kidney or adrenal gland, two soft-tissue organs that can be also moderately colonized in our animal model. This rules out the possibility that the targeted ablation of FKN could non-

specifically affect cell dissemination through blood circulation and reinforces the specificity of the CX₃CR1-FKN pair in regulating the migration of cancer cell to the skeleton.

In light of the *in vivo* results provided by the FKN(-/-) mouse model, we decided to further investigate the involvement of CX₃CR1 in breast cancer metastasis by exogenously expressing this receptor in MDA-436 cells, as we found that these cells fail to express it and migrate to the bone with very low efficiency (**Fig. 2**). In addition, we sought to further dissect the relative impact that CX₃CR1 exerts in adhesion to the endothelium of bone sinusoids and extravasation into the surrounding bone marrow stroma, respectively. We have previously reported that both adhesion and migration of prostate cancer cells can be regulated by FKN *in vitro* ²⁷. However, the targeted deletion of this chemokine removes both the trans-membrane adhesive molecule and the soluble chemoattractant form, and therefore the FKN(-/-) mice could not be used to address this specific issue.

The next series of experiments were then conducted with MDA-436 cells stably expressing either the wild-type form of CX₃CR1 or one of the two following functional mutants of this receptor. The first mutant was generated by inducing a tyrosine to phenylalanine mutation at amino acid 14 of the first extracellular domain of CX₃CR1 (Y14F) ⁴⁰. This mutant was previously characterized for its failure to firmly bind to FKN, most likely because of the inability of phenylalanine to be sulfated, a modification that enhances the binding to this chemokine. Although defective in capture and adhesion, CX₃CR1 (Y14F) is competent in signal transduction, but with a 100-fold decreased affinity to immobilized FKN ⁴⁰. The specific involvement of CX₃CR1 in extravasation was evaluated using a second functional mutant containing an arginine to asparagine mutation at amino acid 128, which is located in the second intracellular loop of CX₃CR1 and in the highly conserved aspartic acid-arginine-tyrosine (DRY) sequence of G-protein coupled receptors ²⁴. Chemoattractant properties of chemokine receptors are dependent on G-protein activation and subsequent ability to transduce downstream signals following stimulation by the appropriate ligand ⁴¹. As the DRY sequence is required for G-protein activation, the R-to-N mutation makes

the receptor incompetent of intracellular signaling ²⁴ and cells expressing the CX₃CR1 (R128N) mutant do not migrate toward FKN, while showing normal binding/adhesion to this chemokine ²⁴. The expression of wild type and mutated forms of CX₃CR1 by MDA-436 cells was verified by western blotting performed on total cell lysates (**Fig. 4A**). In addition, the insertion of each form of the receptor at the plasma membrane of transfected cells was confirmed by isolation of cell surface proteins obtained by biotinylation (**Fig. 4B**). When MDA-436 cells engineered to exogenously express CX₃CR1 were exposed to 50nM soluble FKN *in vitro*, a time-dependent phosphorylation of ERK1/2 was observed. As expected, this downstream signaling was not detected in untransfected cells, as they express minimal levels of CX₃CR1 (**Fig. 5**). The MDA-436 cells expressing the Y14F mutant responded to soluble FKN with a negligible and non-statistically significant activation of the MAPK pathway, most likely as a consequence of their reduced affinity for the chemokine, as discussed above. Finally, the R128N mutant did not transduce downstream signals in MDA-436 cells, confirming its inability in G-protein activation (**Fig. 5**). The role of wild type and functional mutants of CX₃CR1 in regulating the arrest of breast cancer cells to the skeleton was then tested by inoculating MDA-436 cells in mice as untransfected cells or expressing one of the three available forms of the receptor. Animals were sacrificed at either 24 or 72 hours following inoculation, to allow discriminating between the fast adhesion to the bone marrow endothelium and the relatively delayed extravasation into the surrounding stroma ³². After 24 hours, the expression of wild type CX₃CR1 doubled the number of MDA-436 cells detected in the bones of inoculated mice (**Fig. 6A**). This result provides strong support for the role of CX₃CR1 in mediating the arrival of circulating breast cancer cells to the skeleton and further validates the significantly higher ability for skeletal dissemination shown by CX₃CR1-expressing MDA-231 cells as compared to CX₃CR1-negative MDA-436 cells. Cells expressing the CX₃CR1(Y14F) mutant arrested to the bone in numbers comparable to cells lacking the receptor, a clear expression of their inability to bind and adhere to FKN on the luminal side of bone marrow endothelial cells. On the other hand, MDA-436 cells expressing the

CX₃CR1(R128N) mutant arrested to the skeleton with an efficiency comparable to cells expressing the wild type receptor, indicating that extravasation into the marrow stroma required longer than 24 hours from the entering of the blood circulation (**Fig. 6A**). When animals were examined at 72 hours following cell inoculation, the number of MDA-436 cells either lacking or expressing the CX₃CR1 receptor that were detected in the bone was lower than what observed at 24 hours post-inoculation (**Fig. 6B**). This suggests that a significant number of adherent cells fail to extravasate and are eventually dislodged from the endothelial wall. Interestingly, even in these conditions CX₃CR1 still provided MDA-436 cells with a 3-fold higher propensity to remain at the skeleton as compared to cells lacking this receptor. The cells expressing either CX₃CR1(Y14F) or (R128N) mutants failed to show increased lodging in the bone as compared to cells lacking CX₃CR1 expression. This was plausibly due to their inability to initially adhere to the endothelium or migrating toward the soluble FKN produced and released in the bone marrow by stromal cells²⁸, respectively.

Taken together, these results provide definitive support to the idea that the interactions between CX₃CR1 and FKN promote skeletal dissemination of circulating breast cancer cells. Importantly, this observation might be likely extended to other malignant phenotypes, including those deriving from tumors of the prostate gland²⁷. In addition, the role exerted by CX₃CR1 in the arrest to the bone includes the regulation of both cell adhesion and extravasation, in line with the ability of this receptor to interact with both plasma membrane-bound and soluble FKN.

Based on our study, it seems reasonable to postulate that the dissemination to the skeleton of breast cancer cells can be effectively counteracted by interfering with the molecular and functional interactions between the chemokine FKN and its only receptor CX₃CR1. The detection of this receptor in the majority of human breast tissue samples we examined (**Table 1**) suggests that this type of strategy could protect a relevant number of patients from skeletal metastases. In light of the concrete possibility of post-surgery spreading, these results should bolster the synthesis of CX₃CR1 inhibitors and their pre-clinical and clinical testing, to

ultimately conceive new adjuvant therapeutic approaches and promote a paradigm shift in the management of breast cancer patients.

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Table 1.

Tissues	0	0-1	1-2	2-3	Total
Normal	21 (45%)	19 (40%)	6 (13%)	1 (2%)	47
Malignant	43 (21%)	54 (27%)	53 (26%)	52 (26%)	202

Intensity and distribution of CX₃CR1 in normal and malignant breast tissues.

Staining intensity was scored based on a scale in which negative specimens were graded (0) and strongly positive samples were graded (3). To evaluate distribution, tissues with complete lack of staining were scored as (0); tissues prevalently negative for CX₃CR1 displaying some areas of positive staining were scored as (0-1); tissues showing uniform staining for CX₃CR1 were scored as either (1-2) or (2-3), based on the intensity of the signal observed.

Of the 202 malignant samples examined, only 4 were identified as being Ductal Carcinoma In Situ (DCIS): two were scored as (0) and the other two as (0-1) and

(1-2). Additional histopathological cancer subtypes included in the three arrays used in the study were:

Papillary carcinoma: 6 samples that were scored from (1-2) to (2-3);

Invasive lobular carcinoma: 7 samples that were scored from (0) to (1-2);

Infiltrating intraductal carcinoma: 5 samples that were scored from (0) to (2-3).

We compared the tissue samples that stained negative to CX₃CR1 in the normal (21) and malignant (43) groups to all the samples that stained positive to the receptor in the same groups (26 and 159, respectively) and were scored from (0-1) to (2-3). The increase in CX₃CR1 expression observed in cancer samples as compared to normal samples resulted to be very statistically significant with a p value equal to 0.0016.

FIGURE LEGENDS

Figure 1. Immunohistochemistry detection of CX₃CR1 in human breast tissue arrays. Hematoxylin and eosin staining (left) and immunohistochemistry detection of CX₃CR1 (right) in human mammary glands. A positive staining for the chemokine receptor could be observed in epithelial cells from both normal (**A**) and malignant (**B**) breast tissue. The stromal component stained uniformly negative for CX₃CR1. Representative images of 47 normal and 202 malignant tissue cores analyzed. Original magnification, x50.

Figure 2. Expression of CX₃CR1 by human breast cancer cells. Western blotting analysis of CX₃CR1 expression in MDA-231 and MDA-436 breast cancer cell lines. CX₃CR1 was detected in MDA-231 cells, whereas MDA-436 showed negligible levels of the receptor. Cell lysates from the THP-1 human monocytic cell line were included as positive control. Actin was used as a loading control (**A**); single breast cancer cells stably expressing eGFP (arrows) were identified by fluorescence stereomicroscopy in the bone marrow of mice, 24 hours after being inoculated via the left cardiac ventricle (**B**); MDA-231 and MDA-436 cell lines that migrated to the skeleton via the hematogenous route, expressed as mean of cells detected per mouse \pm S.E.M. (**C**). (MDA-231 cells = 4 mice, MDA-436 cells = 8 mice. * p=0.0008).

Figure 3. The arrest of CX₃CR1-expressing cancer cells in the bone of FKN-null mice is significantly impaired. The number of MDA-231 cells detected in the skeleton of transgenic FKN(-/-) mice (n=5) 24 hours after intra-cardiac inoculation is significantly decreased compared to the number of cells detected in normal C56Bl/6 mice (n=4) (**A**); similar results were obtained in analogous experiments conducted with PC3-ML human prostate cancer cells inoculated in FKN(-/-) mice (n=3) and normal controls (n=4) (**B**). The number of cells detected per animal is shown as mean \pm S.E.M. (**-p=0.0002, ***-p<0.0001).

Figure 4. Exogenous expression of wild type and functional mutants of the CX₃CR1 receptor in MDA-436 breast cancer cells. Western blotting analysis of total cell lysates collected from parental MDA-436 cells as well as cells stably expressing the wild type CX₃CR1, the adhesion-impaired Y14F mutant or the chemotaxis-impaired R128N mutant of the receptor. Actin was used as a loading control (**A**); the correct insertion of each exogenously expressed form of the CX₃CR1 receptor on the plasma membrane of transfected MDA-436 cells was confirmed by cell surface protein isolation (**B**). Actin detected in the cytosolic fraction was used as a loading control.

Figure 5. Intracellular signaling of CX₃CR1 and functional mutants exogenously expressed in MDA-436 cells. Western blotting analysis of MDA-436 cells that when exposed to 50nM of human FKN failed to phosphorylate Erk1/2, as expected from the minimal expression of endogenous CX₃CR1. Cells that were engineered to stably overexpress wild type CX₃CR1 (MDA-436+X) showed a significant and time-dependent Erk phosphorylation in response to FKN. Finally, cells expressing the Y14F (MDA436+Y14F) or R128N (MDA436+R128N) functional mutants of the receptor showed a negligible or lack of Erk phosphorylation, respectively (**A**); the intensity levels of Erk phosphorylation obtained from three independent experiments were analyzed by densitometry analysis and normalized using total Erk signals after membrane

stripping to compensate for variations in protein loading among samples (**B**). Data are represented as mean \pm S.E.M. (* $p < 0.003$).

Figure 6. Role of CX₃CR1 in the adhesion and extravasation of breast cancer cells. The number of MDA-436 cells expressing exogenous CX₃CR1 that were detected in the bone of mice 24 hours after intracardiac inoculation was significantly larger than that of untransfected MDA-436 cells. The expression of the adhesion-defective Y14F mutant of the receptor failed to increase the arrival of MDA-436 cells to the skeleton, whereas the chemotaxis-defective R128N mutant was still able to promote arrival to bone equally to the wild type CX₃CR1 (**A**); when animals were inspected at 72 hours post-inoculation, the difference in bone arrival between parental and CX₃CR1-expressing MDA-436 cells was even more significant than what observed after 24 hours. Also at this later time point, cells expressing the Y14F adhesion-defective mutant were detected in numbers similar to the untransfected cells. Finally, the cells expressing the R128F mutant, despite their ability to adhere normally to the bone marrow endothelium at 24 hours, failed to extravasate and were then detected in numbers similar to the untransfected cells (**B**). Between four and seven mice were used for the analysis of parental and different CX₃CR1-expressing MDA-436 cells migrated to the skeleton at each time point. Data are reported as mean \pm S.E.M. (*- $p \leq 0.03$, **- $p \leq 0.006$, ***- $p < 0.0001$).

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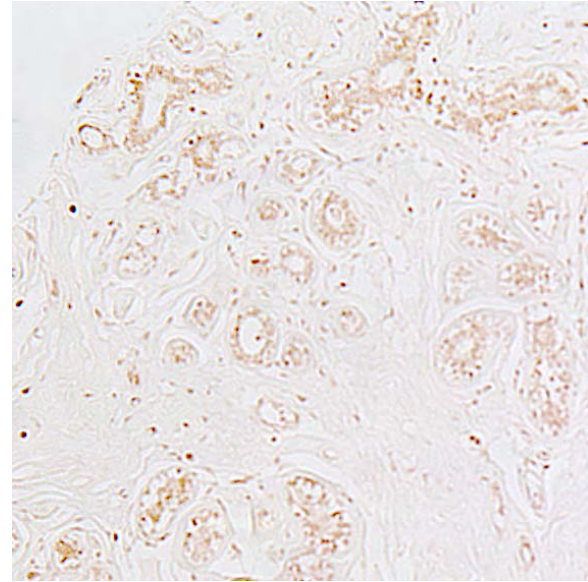
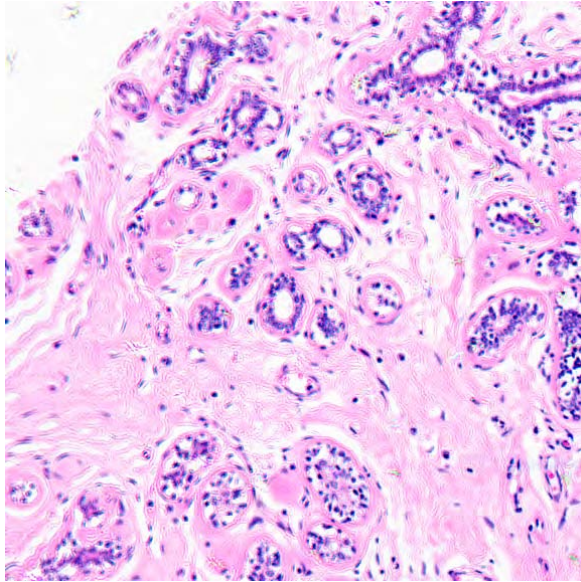
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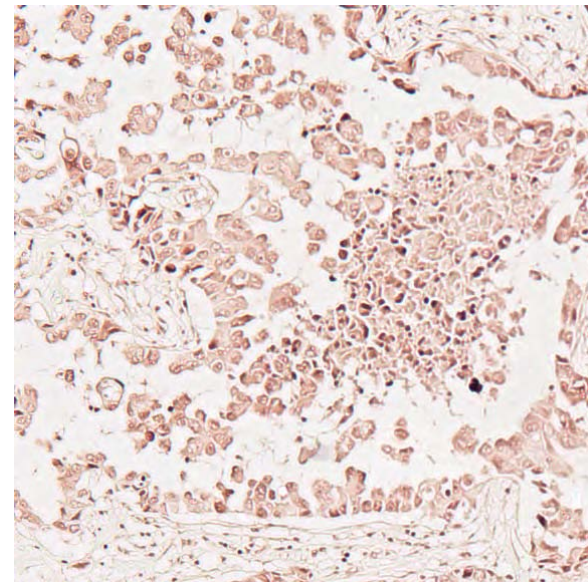
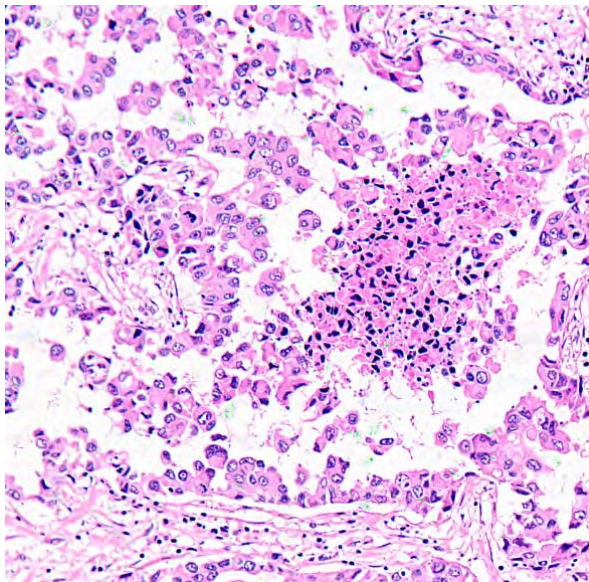
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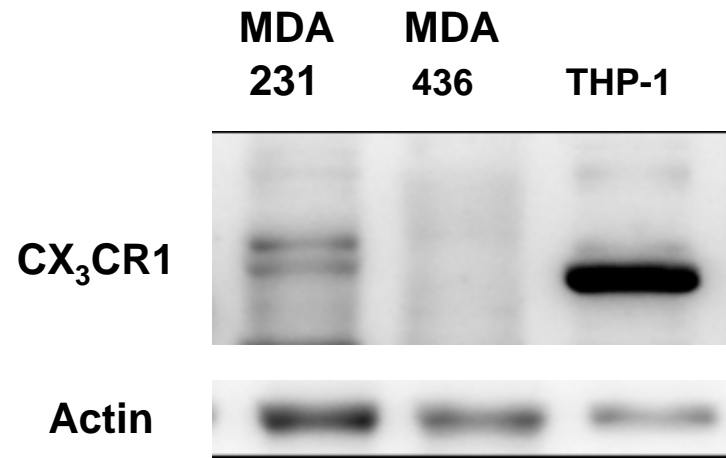


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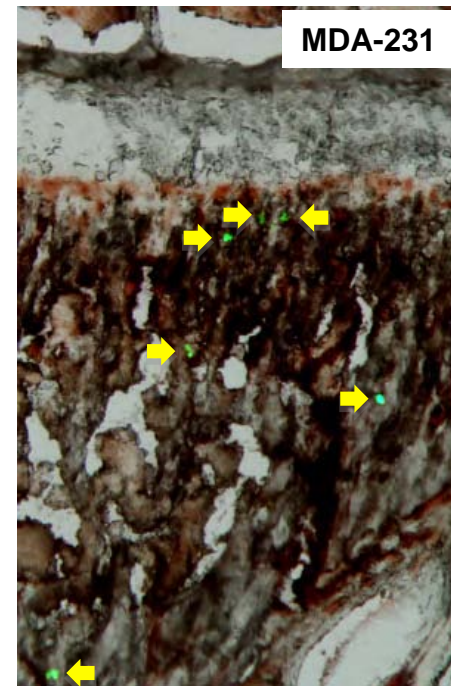
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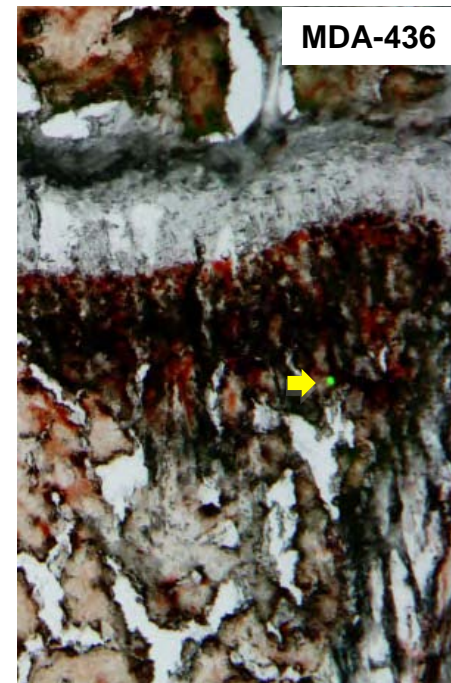
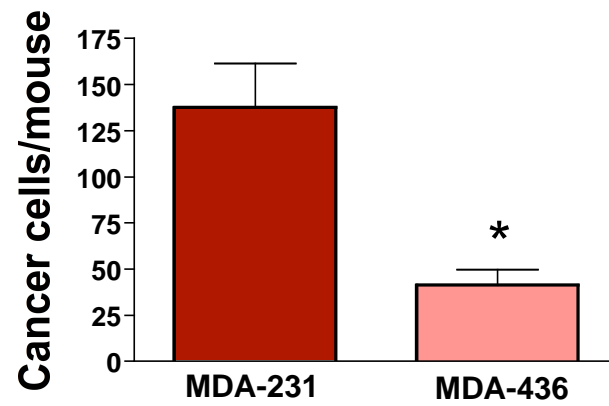
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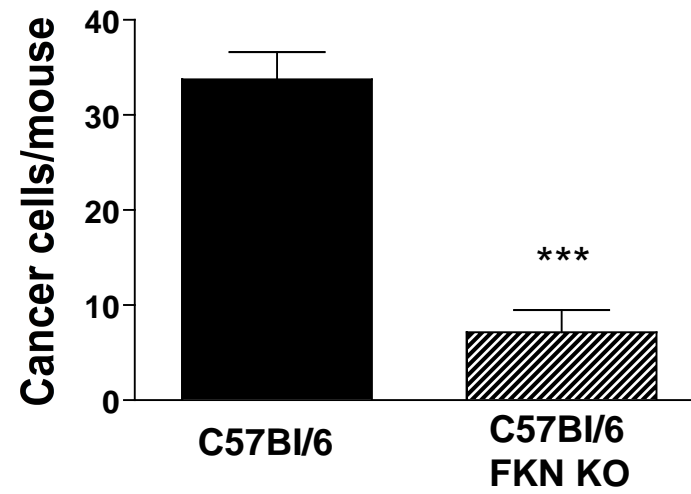
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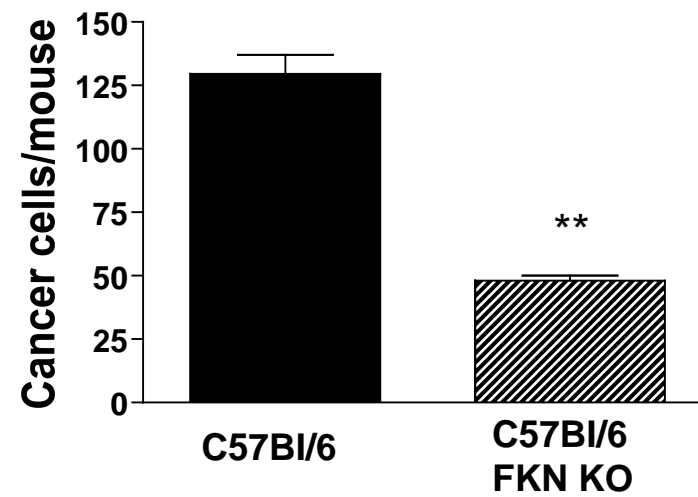
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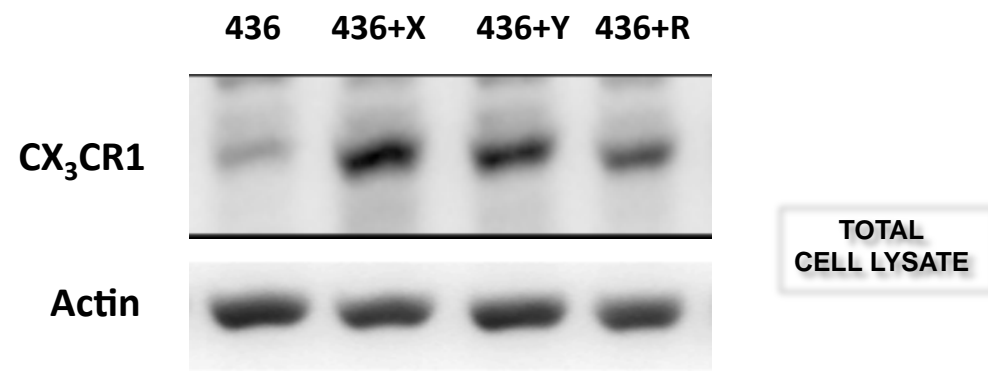


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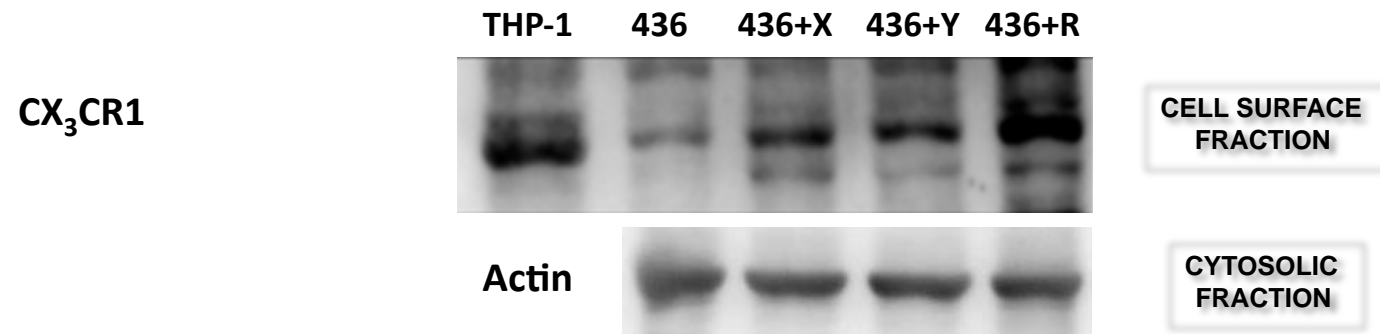
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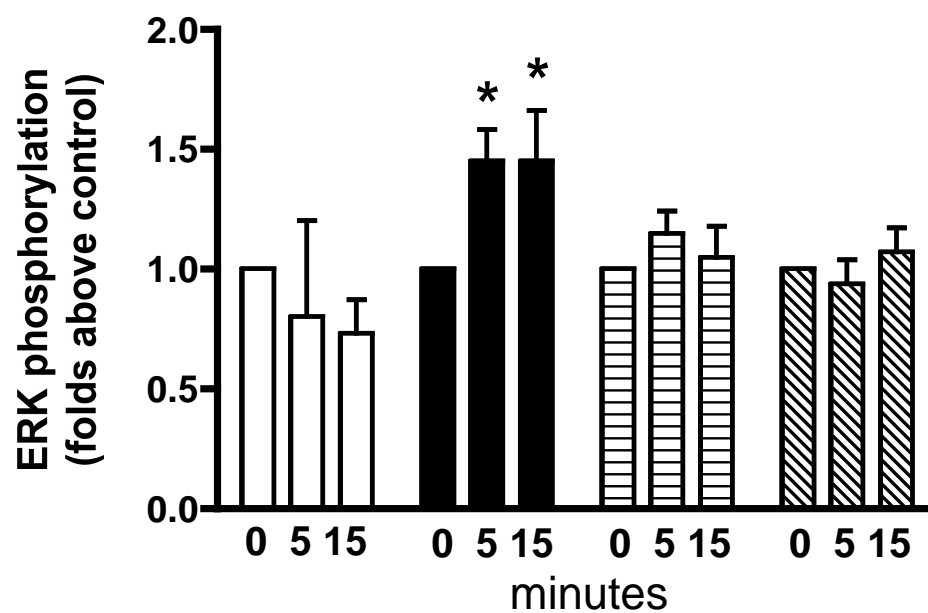
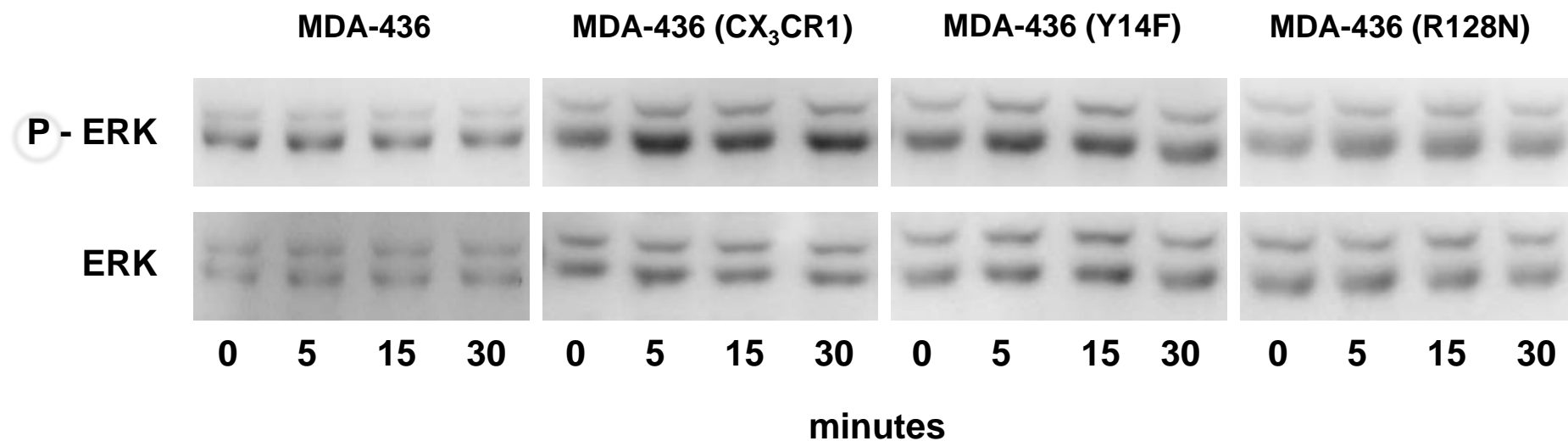
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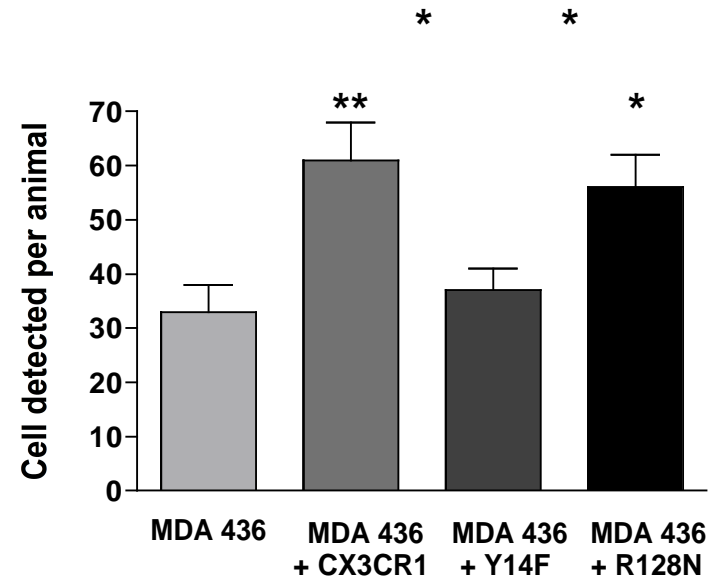
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MDA-436 MDA-436 (Y14F)
 MDA-436 (CX₃CR1) MDA-436 (R128N)

A**B**